

Pathogenesis of peritonitis. I. The effect  
of *Escherichia coli* and  
hemoglobin on  
peritoneal absorption

ROBERT M. FILLER

CAPTAIN, MC, USAR

H. KENNETH SLEEMAN, PH.D.

WASHINGTON, D. C.

From the Division of Surgery, Walter Reed Army

Institute of Research, Walter Reed Army

Medical Center

MAY 17 1967

A

AD 651843  
P  
Previous work has demonstrated that an intraperitoneal injection of a mixture of *Escherichia coli* and hemoglobin produces a lethal peritonitis, whereas *E. coli* or hemoglobin injections singly do not. Characterization of the role of hemoglobin in this situation is highly desirable. Among many postulates is the possibility that hemoglobin retards the clearance of organisms from the peritoneal cavity. This, in turn, would isolate the organisms from many of the host's defense mechanisms to which they would otherwise be exposed upon their departure from the peritoneal cavity.

To test this hypothesis, two experimental approaches were utilized to determine the clearance of bacteria from the peritoneum of animals subjected to *E. coli* peritonitis.

In the first series of experiments, bacterial counts of peritoneal exudate were made at selected intervals following intraperitoneal inoculation of a known number of organisms. This would reflect not only the number of bacteria cleared from the peritoneum but also the number of new organisms which emerged in vivo, subsequent to the inoculation.

In the second series of experiments, the absorption of dye-tagged protein from the peritoneal cavity was determined following the establishment of the infection. Previous studies<sup>3, 5, 6, 10, 11</sup> have indicated that large

molecular substances and colloidal particles, such as bacteria, are similarly absorbed from the peritoneum through the diaphragmatic lymphatics. Therefore, a study of the peritoneal absorption of a nonliving macromolecular substance would provide data to substantiate the bacteriological results obtained in the first series of experiments. Moreover, if one uses a material which can be simply and accurately recovered and measured in the blood, one can estimate peritoneal absorption by the appearance of this substance in the blood. In this study, albumin of normal plasma was tagged with Evans blue dye in vitro. This tagged plasma was injected intraperitoneally, and its appearance measured in the blood of animals which were previously inoculated with *E. coli* organisms in the presence and absence of hemoglobin.

#### METHODS

**Experiment I.** The absorption of bacteria was studied in this experiment by direct bacterial counts of the peritoneal exudate. Twenty Walter Reed strain male white rats, weighing between 225 and 250 grams, were used. Animals were divided into two equal groups, half of which received an injection of *E. coli* in nutrient broth, the other half an injection of *E. coli* plus 4 Gm. percent of hemoglobin in nutrient broth. The principles of laboratory animal care as promulgated by the National Society for Med-

Received for publication May 20, 1966.

ical Research were observed in all experiments.

The *E. coli* suspensions were prepared from stock culture of *E. coli* 0111:B4 by inoculating these organisms in nutrient broth and incubating them at 37° C. for 18 hours. The *E. coli*-hemoglobin mixtures were similarly prepared. In this case, washed red blood cells which were obtained from normal rats were lysed with distilled water and utilized as a source of hemoglobin. The lysed red cells were placed into nutrient broth and the concentration of hemoglobin in the broth was adjusted to 4 Gm. percent as originally described by Davis and Yull.<sup>7</sup> The hemoglobin-broth mixture was inoculated with the stock *E. coli* and incubated for 18 hours at 37° C. Animals were injected with 5 c.c. per kilogram of the appropriate *E. coli* suspension and, each hour for five hours, two animals from each group were put to death. An additional two animals were observed until death or for 22 hours in the case of the nonlethal inoculation.

Bacterial counts were performed on the inocula just prior to intraperitoneal injection by serial tube dilution and plating (blood agar) in triplicate. The total number of bacteria remaining within the peritoneum at the time of death was determined by the method of Cohn.<sup>4</sup> Normal saline (20 c.c.) was injected into the rat peritoneum. By appropriate manipulations, the peritoneal cavity was agitated for a period of two minutes to insure thorough mixing of the injected saline with the peritoneal exudate. A 1 c.c. sample was then withdrawn serially diluted, and bacterial counts were performed by standard plating technique. To obtain the total bacterial count, the number of bacteria present in the 1 c.c. sample was multiplied by a dilution factor of 20. Since the total exudate in the peritoneal cavity at the time of death never exceeded 2 c.c., and was usually about 1 c.c., the maximum error by this method was 10 percent.

Viable bacterial counts were also obtained on rat blood specimens. Heart's blood was obtained through the open chest at the time of death; care was taken to eliminate

possible contamination from the contents of the peritoneal cavity. Bacterial counts were performed by tube dilution and plating techniques, as described for peritoneal fluids.

**Experiment II.** The absorption of plasma tagged with Evans blue from the peritoneal cavity of rats was studied in this experiment. Tagged plasma was prepared by a method described by Courtice and Steinbeck.<sup>8</sup> Plasma obtained from normal rats was tagged by the addition of 1 c.c. of 0.5 percent aqueous solution of Evans blue dye to each 5 c.c. of plasma. All of the Evans blue dye was attached to plasma proteins as shown by precipitation of the proteins with trichloroacetic acid.

Walter Reed white rats weighing between 225 and 250 grams were placed in three groups of ten animals each. The rats in Group 1 were injected intraperitoneally with nutrient broth and served as controls; those in Group 2 received an intraperitoneal injection of *E. coli* organisms without hemoglobin; and those in Group 3, *E. coli* organisms with 4 Gm. percent hemoglobin. The inoculum was 5 c.c. per kilogram in all three groups. Two hours following these initial injections, 5 c.c. per kilogram of plasma tagged with Evans blue dye was injected into the peritoneum of the animals. Animals were anesthetized with Nembutal, 1½ hours following the tagged plasma injection and 3½ hours following the initial intraperitoneal challenge, and 5 c.c. of blood was withdrawn from the aorta. A portion of the blood sample was placed in a microhematocrit tube for hematocrit determinations, and the remainder of the sample was centrifuged for 20 minutes at 3,000 r.p.m. to separate the plasma.

The concentration of blue dye (tagged plasma) in the blood sample was determined spectrophotometrically (625 mμ) by microcuvettes with a 1 cm. light path. Samples were read against a saline blank and concentration was calculated from a standard curve. Samples which contained excessively high dye concentration were diluted with saline.

**Experiment III.** This study was performed to determine the rate of absorption

of tagged-plasma dye from the peritoneum of a single animal at various times following the induction of peritonitis. Because of the small size of the rat and the amount of blood necessary to perform a single plasma Evans blue concentration, multiple samplings from the rat were impractical. Therefore, the dog was used to study the relation of the uptake of tagged plasma to time.

Mongrel dogs weighing between 10 and 15 kilograms were anesthetized with sodium Pentothal (0.5 c.c. per kilogram) after premedication with 0.7 mg. of atropine intravenously. The external jugular vein was exposed and a large polyethylene cannula was passed down into the superior vena cava. The tube was tunneled beneath the subcutaneous tissues to the back of the dog's neck. The end of the tube was capped with a three way stopcock and filled with heparinized saline solution. This tube was used for obtaining future blood sampling. A trochar was inserted into the left upper quadrant of the dog's abdomen. After removal of the stylet, a large-bore polyethylene tube was inserted into the peritoneal cavity, the trochar was removed, and the polyethylene tube was anchored to the abdominal wall. The animals were allowed to recover completely from anesthesia before studies were conducted.

The dogs were then divided into five groups of four dogs each. Through the indwelling peritoneal tube each animal received an intraperitoneal injection of 5 c.c. per kilogram body weight of the following:

Group 1, sterile nutrient broth; Group 2, sterile normal saline; Group 3, sterile nutrient broth with 4 Gm. percent hemoglobin; Group 4, an 18 hour culture of *E. coli*; Group 5, an 18 hour culture of *E. coli* which contained 4 Gm. percent hemoglobin. Hemoglobin was derived from lysed dog red cells as described in Experiment I, and the *E. coli* organisms were obtained from the stock cultures and incubated as described above.

Two hours following these initial injections, 5 c.c. per kilogram of plasma tagged with Evans blue dye was injected through the indwelling polyethylene intraperitoneal tube. No leak of tagged plasma or peritoneal exudate occurred. The tagged plasma was prepared as in the rat experiments, except that normal dog plasma was used.

Following the intraperitoneal injection of tagged plasma, 3 c.c. blood samples were obtained from the superior vena cava catheter and analyzed for hematocrit and Evans blue dye concentrations as in Experiment II. Samples were drawn every hour for 5 hours, and, in several of the animals, collections were made at 8 and 24 hours after the intraperitoneal injection of tagged plasma.

## RESULTS

**Experiment I.** Bacterial counts following the intraperitoneal injection of *E. coli* organisms, with and without hemoglobin, are compared in Table I. There was an initial fall in total viable bacteria in each of these two groups. However, after the initial drop

**Table I.** Bacteria present in the rat's peritoneal cavity following intraperitoneal injection of *E. coli* with and without hemoglobin

	Time after intraperitoneal injection (hr.)	Experiment condition	
		<i>E. coli</i> + hemoglobin	<i>E. coli</i>
Number of bacteria injected	0	$1.58 \times 10^{11}$	$1.43 \times 10^{11}$
Number of bacteria in peritoneal cavity (each figure represents average value of 2 animals)	1	$0.86 \times 10^9$	$75.0 \times 10^8$
	2	$2.9 \times 10^9$	$19.3 \times 10^8$
	3	$4.7 \times 10^9$	$9.8 \times 10^8$
	4	$6.1 \times 10^9$	$8.5 \times 10^8$
	5	$10.2 \times 10^9$	$10.8 \times 10^8$
	22	$1.0 \times 10^9$	$1.0 \times 10^8$

in counts, the animals which received *E. coli* with hemoglobin remained relatively stable; more than  $10^4$  organisms were found in the peritoneal cavity of all the animals. There seems to be a slowly progressive increase in bacterial numbers in this group throughout the remainder of this 5 hour observation. Although the initial drop in bacterial count in the dogs which received *E. coli* without hemoglobin was the greatest drop that occurred in the 5 hour period, the number of bacteria present in the peritoneal cavity after 3 hours was approximately 1,000-fold less in this group as compared to the group with *E. coli*-hemoglobin peritonitis, and at 22 hours (or death) this difference was even more marked. Blood cultures which were obtained from intracardiac puncture revealed no significant differences between these two groups of animals during the initial 5 hour period of observation. Counts ranging from  $5 \times 10^3$  to  $4 \times 10^5$  organisms per cubic centimeter of blood were found in the blood of all animals put to death 1 to 5 hours after intraperitoneal challenge. At 22 hours (or death), only those animals dying had a bacteremia.

**Experiment II.** The absorption of dye-tagged protein from the peritoneum following the induction of experimental peritonitis is shown in Table II. The concentration of Evans blue dye in the plasma of these three groups of animals was quite different. There

is marked retardation of peritoneal absorption in animals subjected to *E. coli*-hemoglobin (Group 3) peritonitis as compared to those animals challenged with *E. coli* without hemoglobin (Group 2). The absorption of dye-tagged plasma in Group 2 was more than ten times greater than that found in Group 3. The highest plasma dye concentrations were, as expected, in Group 1, the nutrient broth control. The hematocrits were remarkably constant within each of these three groups. Hematocrit values ranged from 43 to 46 percent in Group 1 animals, 41 to 47 percent in Group 2 animals, and 47 to 54 percent in Group 3 animals.

**Experiment III.** The results of serial determinations of tagged plasma absorption in dogs for a 5 hour period starting 2 hours after the intraperitoneal challenge are noted in Fig. 1. In this figure, Group 1 nutrient broth controls are compared with animals challenged with *E. coli* and *E. coli*-hemoglobin solution. Animals that were challenged with just hemoglobin or saline are not listed in Fig. 1, but the plasma-dye concentrations of these animals were identical to those of the Group 1 animals which had received only nutrient broth as a challenge. These results parallel those of the Experiment II, and demonstrate that a decrease in peritoneal absorption occurred when hemoglobin was added to the *E. coli* suspension,

**Table II.** Rat plasma dye concentration  $1\frac{1}{2}$  hours after intraperitoneal injection of dye and  $3\frac{1}{2}$  hours after intraperitoneal injection of *E. coli* with and without hemoglobin

Animal No.	Plasma Evans blue dye concentration		
	Group I (nutrient broth control) (mg./L.)	Group II ( <i>E. coli</i> ) (mg./L.)	Group III ( <i>E. coli</i> + hemoglobin) (mg./L.)
1	36.40	12.00	0.65
2	26.40	18.30	3.75
3	24.40	6.60	0.65
4	16.80	15.90	0.25
5	25.20	20.10	1.00
6	28.80	12.90	1.30
7	31.10	11.40	2.05
8	24.00	21.30	1.65
9	19.50	15.30	0.50
10	29.40	23.40	0.90
Mean $\pm$ S.E.	26.20 $\pm$ 2.98	15.72 $\pm$ 1.55	1.27 $\pm$ 0.31

whereas nutrient broth, saline, and hemoglobin alone had little effect. The absorption was subjected to statistical analysis, and a comparison of the mean absorption during this 5 hour period between the five groups based on regression analysis was 18.9 mg. per liter with nutrient broth, 17.1 mg. per liter with hemoglobin, 17.0 mg. per liter with saline, 11.4 mg. per liter with *E. coli*, and 5.4 mg. per liter with *E. coli* plus hemoglobin. Evans blue dye concentrations in the plasma at 8 hours and 24 hours after challenge are not listed in Fig. 1, but results showed that the plasma concentrations of dye did not increase significantly after the

5 hour period in Groups 1, 2, or 3. This is after dye concentration reached an average of 25 mg. per liter. In Groups 4 and 5, in which peritoneal absorption was decreased, a persistent increase in dye concentration occurred, so that after 24 hours there were no significant differences in plasma dye concentrations between any of the groups. Hematocrit values were quite similar in all groups of dogs, and ranged from 40 to 50 percent.

### DISCUSSION

Previous studies with rats and dogs have demonstrated that an intraperitoneal injec-

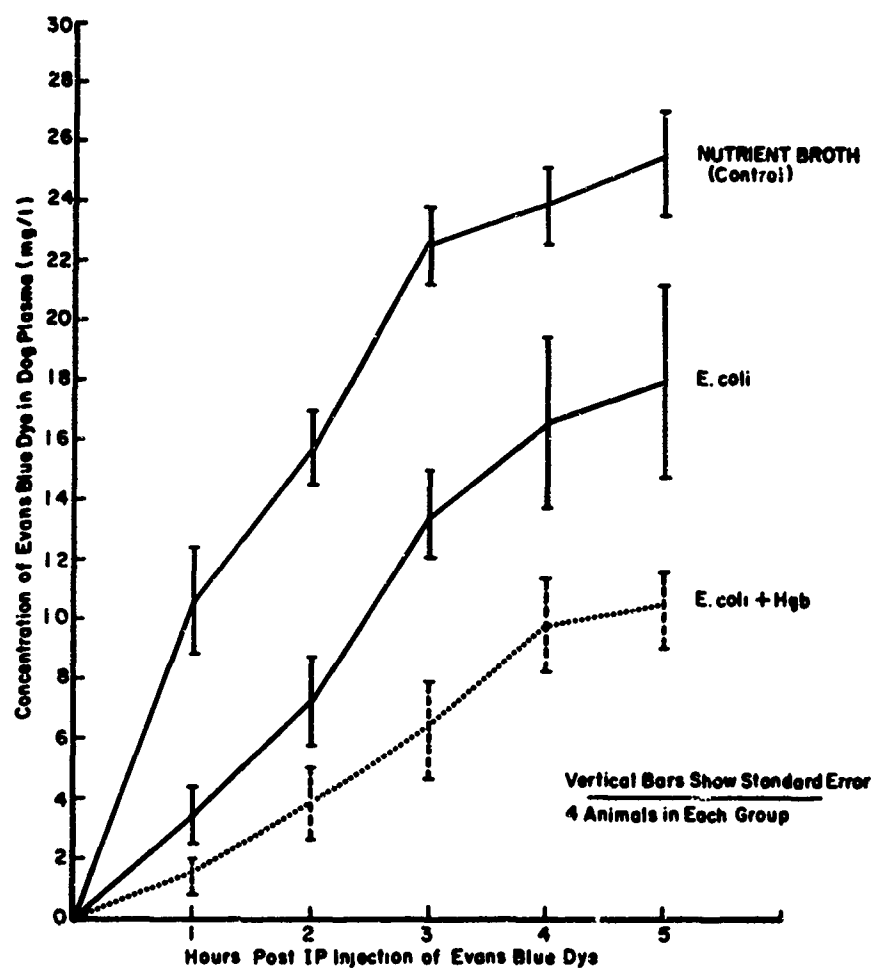


Fig. 1. Peritoneal dye absorption in dogs following intraperitoneal injection of *E. coli* with and without hemoglobin.

tion of *E. coli* ( $10^8$  to  $10^{11}$  organisms) is not lethal. If, however, hemoglobin at concentrations of 4 Gm. percent or greater is added to the inoculum, approximately 70 percent of the animals die in 22 to 24 hours.<sup>7</sup> The observed lesions are similar to those found following the intravenous injection of purified bacterial endotoxin. Defining the role of hemoglobin in this system has been the purpose of these and other studies.

The results of the present experiments indicate that absorption of bacteria from the peritoneal cavity is reduced when a lethal peritonitis is induced by the addition of hemoglobin to an otherwise nonlethal *E. coli* intraperitoneal inoculation.

Direct bacterial counts of organisms remaining in the peritoneum 5 hours after intraperitoneal bacterial injections reveal that the addition of hemoglobin to the inoculum results in the recovery of a thousand times more bacteria than when hemoglobin is not added. Since viable organisms were counted by our method, one might also interpret these findings to mean that no change in bacterial absorption occurred, but that hemoglobin retarded local defense mechanisms allowing unopposed bacterial growth in the animals challenged with hemoglobin and bacteria. Steinberg,<sup>10</sup> however, has shown that phagocytosis provides the major means of bacterial destruction in the peritoneum, and Balch and Kelly<sup>2</sup> found no alteration of the bactericidal capacity of polymorphonuclear leukocytes against *E. coli* or *Staphylococcus albus* in the presence of hemoglobin. Davis and Yull<sup>7</sup> have suggested that hemoglobin may block the reticuloendothelial system, but confirmatory studies have not been reported.

The results of Experiment II further support the thesis that a decrease in peritoneal absorption of particulate matter is responsible for the observed differences in bacterial counts. A comparison of peritoneal absorption of tagged protein in rats with established *E. coli* peritonitis to the absorption in rats with *E. coli*-hemoglobin peritonitis reveals that plasma Evans blue concentrations 90 minutes after the intraperitoneal injection

of tagged protein were ten times higher in the former group. Since plasma concentration of tagged protein, rather than total amounts of tagged protein, was used as a measure of absorption, one might argue that differences in circulating plasma volumes between the two groups might account for the measured differences in concentration. It is well known that peritonitis causes an exudation of plasma into the peritoneum and, the more severe the peritonitis, the greater the amount of exudate. Utilizing the average hematocrit values found in the two groups of animals, and assuming no differences in red cell mass, calculations show that the circulating plasma volume in the animals with *E. coli*-hemoglobin peritonitis was about 20 percent (or 2 c.c. in 250 Gm. rats) less than in those with *E. coli* peritonitis. This calculated figure correlates well with the difference in peritoneal exudate actually found when the quality of exudate is compared in several animals from each group. The interpretation of this difference in plasma volumes suggests that: (1) the observed concentration of tagged protein in animals with *E. coli*-hemoglobin peritonitis represents even less total absorption of tagged material than in animals with *E. coli* peritonitis, and (2) even if plasma lost as exudate into the peritoneum of animals with *E. coli*-hemoglobin peritonitis contained tagged protein at its maximal concentration, this 20 percent difference in volume could hardly be expected to account for the observed 1,000 percent difference in plasma concentrations of tagged protein.

The methods utilized in Experiments I and II both reveal that the addition of hemoglobin to intraperitoneal *E. coli* injections causes a decrease in peritoneal absorption; however, the degree of decrease was quite different for each method. The bacterial counts revealed a thousandfold decrease (Table I) whereas, the tagged plasma absorption was decreased tenfold (Table II). This might be construed to mean that bacteria and protein are absorbed by different mechanisms. However, it seems far more likely that this discrepancy is due to bacterial

multiplication. Each bacterial cell which is not absorbed from the peritoneum and remains viable has the ability to multiply about every 20 minutes. Therefore, the number of bacteria observed in the peritoneum depends not only on the number absorbed but also on the number of new organisms formed. One bacterial cell not absorbed from the peritoneum represents eight bacterial cells in the peritoneal cavity 60 minutes later. On the other hand, only the tagged protein which is added to the peritoneum retains the Evans blue dye, and the total number of tagged particles remains constant. One tagged particle not absorbed represents one tagged particle 60 minutes later.

Quantitative bacterial counts in the blood of animals in Experiment I did not reflect the changes in peritoneal absorption of organisms. This is not surprising, since blood levels of bacteria represent not only those organisms which are added to the circulation from the peritoneum but also those bacteria which are removed and then added back to the circulation from the tissues.

Evidence that bacteria are absorbed from the peritoneal cavity in a fashion similar to that of protein can be found in numerous previous investigations. Early studies showed that saline solutions were absorbed directly from the general peritoneal cavity into the blood.<sup>5, 9</sup> Soluble ion exchange between the blood and peritoneum followed the laws of diffusion and osmosis and was not driven by other forces, chemical or mechanical.<sup>3, 10</sup> Peritoneal absorption of particulate colloidal substances and large molecules involves different mechanisms. Many workers<sup>1, 6, 10</sup> have shown that all these larger particlets are absorbed primarily through the diaphragmatic peritoneum and gain entrance into the diaphragmatic lymphatics. Stomas in the diaphragmatic peritoneum have been described<sup>1</sup> through which large particles pass on their way to the lymphatics. After entering the subserosal lymphatic plexuses, particles enter the diaphragmatic lymph capillaries and eventually transverse the anterior mediastinum to enter the bloodstream via the right lymphatic or thoracic duct.

The present studies do not explain why the addition of hemoglobin to the *E. coli* suspensions hinders peritoneal clearance of organisms. As noted in Experiment III, when hemoglobin without organisms is introduced into the peritoneum, the absorption of tagged protein particles is no different from the absorption of sterile nutrient broth. *E. coli* organisms alone, on the other hand, will depress tagged protein absorption. Somehow the combination of *E. coli* and hemoglobin exerts an even greater depressive effect. We have thought that perhaps particle size or particle numbers in relation to the blocking of the stomas in the diaphragmatic peritoneum may be influential. This is the subject of a subsequent report.

Many mechanical factors can also influence peritoneal absorption.<sup>10, 11</sup> For example, general anesthesia, situations where respiratory excursion is decreased, and positioning so that gravity favors the flow of material away from the diaphragm all may result in decreased peritoneal absorption. In the present study there was no apparent difference in respiration between the groups of animals. The animals were all unanesthetized at the time of induction of peritonitis and were allowed to rest in their cages in the prone position. It is possible that hypotension might change peritoneal absorption, but, in other studies in our laboratory utilizing this same model, no hypotension was noted during the first 8 hours following the induction of *E. coli*-hemoglobin peritonitis.

In addition to the changes in absorption of large particles, such as protein and bacteria, studies by Miller<sup>8</sup> demonstrate changes in the absorption of smaller soluble particles when the peritoneum is inflamed. In contrast to the depressed absorption of large particles which we have observed, absorption of readily diffusible compounds such as glucose and the dye bromphenol blue is actually accelerated.

Regardless of the exact mechanism, changes in peritoneal absorption may have much significance in the pathogenesis of lethal peritonitis. Decreased absorption of bacteria will allow bacterial multiplication in

a portion of the body exposed only to local defense mechanisms rather than to the entire defense mechanisms of the host. Under such circumstances it is possible that large amounts of bacterial endotoxin and other bacterial products will be produced. If, as suggested by Miller, there is a continued or even accelerated peritoneal absorption of these soluble toxins, then a fatal outcome would be anticipated.

### SUMMARY

The absorption of bacteria and colloidal protein from the peritoneal cavity was reduced when lethal peritonitis was induced by the addition of hemoglobin to an otherwise nonlethal *E. coli* intraperitoneal inoculation. Decreased peritoneal absorption was demonstrated by determining, at selected times after infection, (1) the number of bacteria contained in the peritoneal cavity, and (2) the absorption of dye-tagged protein from the peritoneal cavity into the blood. The lethality of the *E. coli*-hemoglobin inoculum was attributed to the decreased clearance of bacteria from the peritoneal cavity, which permitted their continued growth and production of soluble, absorbable toxins.

### REFERENCES

1. Allen, L., and Weatherford, T.: Role of fenestrated basement membrane in lymphatic absorption from peritoneal cavity, *Am. J. Physiol.* 19: 551, 1959.
2. Balch, H. H., and Kelly, D.: Effect of hemoglobin on bactericidal capacity of human leukocytes, *J. Surg. Res.* 5: 262, 1965.
3. Boen, S. T.: Peritoneal dialysis in clinical medicine. Springfield, Ill., 1964, Charles C. Thomas, Publisher, Chap. 1.
4. Cohn, Z. A.: Determinants of infection in the peritoneal cavity. I. Response to and fate of *Staphylococcus aureus* and *Staphylococcus albus* in the mouse, *Yale J. Biol. & Med.* 35: 12, 1962.
5. Courtice, F. C., and Steinbeck, A. W.: The rate of absorption of heparinized plasma and of 0.9 p.c. NaCl from the peritoneal cavity of the rabbit and guinea pig, *Australian J. Exper. Biol. & M. Sc.* 33: 171, 1950.
6. Courtice, F. C., and Steinbeck, A. W.: The lymphatic drainage of plasma from the peritoneal cavity of the cat, *Australian J. Exper. Biol. & M. Sc.* 28: 161, 1950.
7. Davis, J. H., and Yull, A. B.: A toxic factor in abdominal injury. II. The role of the red cell component, *J. Trauma* 4: 84, 1964.
8. Miller, G. R.: The influence of inflammation on the absorption of substances of varied diffusibility, *J. Exper. Med.* 57: 619, 1938.
9. Starling, E. H., and Tubby, A. H.: On absorption from the secretion into the serous cavities, *J. Physiol.* 16: 140, 1894.
10. Steinberg, B.: Infections of the peritoneum, New York, 1949, Harper and Brothers, Chap. II.
11. Zinsser, H. H., and Pryde, A. W.: Experimental study of physical factors, including fibrin formation influencing the spread of fluids and small particles within and from the peritoneal cavity of the dog, *Ann. Surg.* 136: 818, 1952.